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Homoisoleucine: A translationally-active leucine surrogate of expanded hydrophobic surface area

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Non-canonical amino acids (ncAAs) provide useful tools for the investigation and control of protein behavior.[1] Several laboratories have used ncAAs to explore the role of hydrophobic forces in stabilizing proteins, including prion proteins, T4 lysozyme, chloramphenicol acetyltransferase, green fluorescent protein, and coiled-coil and helix-bundle proteins.[2] Here we examine the consequences of introducing the leucine surrogate (2*S*,4*S*)-2-amino-4-methylhexanoic acid (homoisoleucine, Hil, **2**)[2a] into the coiled-coil peptide A1 (Figure 1).[3] A1 contains six heptad repeats, designated (*abcdefg*), that mediate self-association of the peptide in aqueous solutions. Previous studies have shown that replacement of leucine by (2*S*,4*R*)-trifluoroleucine (Tfl, **3**) or hexafluoroleucine (Hfl, **4**) at the *d* positions of the heptad repeats leads to substantial stabilization of the coiled-coil structure of A1,[2e, 2f, 2h] presumably through enhanced hydrophobic interactions between fluorinated peptide strands. Similar results have been obtained in other fluorinated coiled-coil and helix-bundle systems.[4]

Whitesides and coworkers have pointed out that hydrocarbons and fluorocarbons exhibit equivalent “intrinsic” hydrophobicities when changes in molecular surface area are taken into account.[5] Marsh and coworkers have argued that the “efficient packing” of the bulkier fluorinated amino acids in helix bundle cores can be more important than fluorination per se.[6] Because the molecular surface areas of Hil and Tfl are nearly identical (and larger than that of Leu by 14–19 Å^2),[7] we imagined that replacement of Leu by Hil might stabilize coiled-coil peptides such as A1.

We first focused our attention on the translational activity of Hil in bacterial cells. Schultz and coworkers reported incorporation of Hil into proteins via chemical misacylation of suppressor tRNA and *in vitro* translation,[2a] but we are unaware of previous studies of incorporation of Hil into cellular proteins. *E. coli* strain LAM1000 (a previously reported leucine auxotroph) was co-transformed with expression plasmid pA1EL and repressor plasmid pREP4.[2f] pA1EL codes for both the protein A1 and a constitutively expressed copy of the *E. coli* leucyl-tRNA synthetase (LeuRS) gene. Protein expression was induced in

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minimal medium depleted of Leu and supplemented with Hil (see Supporting Information for details). Electrophoretic analysis of whole-cell lysates indicated high-level protein expression in media containing as little as 0.25 mM L-Hil, a concentration comparable to the concentrations of canonical amino acids in minimal media.

ATP-PP_i exchange assays confirmed that Hil is activated by the *E. coli* LeuRS, albeit at a rate substantially lower than that characteristic of the natural substrate (Table 1). The reduced rate of activation of Hil is consistent with our observation that Hil supports high-level protein expression only when LeuRS is over-expressed in the bacterial host.

A1 samples containing Leu and Hil were purified from 25-mL cultures in yields of 15.9 ± 2.5 mg L^{-1} and 10.2 ± 1.1 mg L^{-1}, respectively. Liter-scale expression and purification improved the yields of A1 sequence variants containing Hil two- to threefold. Liquid chromatography/mass spectrometry (LC/MS) indicated replacement of at least 97% of Leu by Hil (see “Determination of Amino Acid Replacement Levels” in the Supporting Information).

Figure 2A shows circular dichroism spectra of 10 mM solutions of the Leu- (Leu-A1) and Hil- (Hil-A1) forms of A1. Strong minima at 208 and 222 nm confirm that both proteins assume α-helical structures; K2D2[9] analysis indicates helical contents of 60–69%, consistent with the fact that the putative heptad repeats constitute 57% of the peptide sequence. A1 is expected to exist primarily as dimers under the conditions employed here.[10]

Replacement of Leu by Hil increases the denaturation temperature of A1, as expected. Figure 2B shows the molar ellipticities at 222 nm of solutions of Leu-A1 and Hil-A1 as functions of temperature. Fitting the CD data to a model of a two-state transition between folded dimer and unfolded monomer states[11] yielded melting temperatures of 58.7±0.2°C and 75.8±0.1°C for Leu-A1 and Hil-A1, respectively.

Table 2 compares the extent to which the thermal denaturation temperature of A1 is elevated by replacement of Leu by bulkier hydrocarbon and fluorocarbon surrogates. Replacement of Leu by Hil raises T_m by 17°C, as compared to 10°C for replacement of Leu by (2*S*,4*R*)-Tfl and 22°C for replacement by Hfl. Expansion of hydrophobic side chain volume at the *d*-position of the heptad repeat constitutes an effective strategy for stabilization of coiled-coil peptides, irrespective of the hydrocarbon or fluorocarbon character of the side chain.

We do not mean to suggest similar molecular origins for the hydrophobic properties of hydrocarbons and fluorocarbons. Although Hil and Tfl behave similarly with respect to elevation of the melting temperature of A1, other experiments suggest important differences in the behavior of water adjacent to hydrocarbon and fluorocarbon side chains. Recent studies via ultrafast spectroscopy indicate a marked slowing of water motions upon replacement of Leu by Tfl at solvent-exposed sites[12]. In contrast, replacement of Leu by Hil is accompanied by increased rates of solvent reorganization. Much remains to be done to elucidate the origins of hydrophobic effects in proteins and other molecular systems.

In conclusion, we find that Hil serves as an effective surrogate for Leu with respect to protein translation in bacterial cells, and that replacement of Leu by Hil leads to substantial stabilization of recombinant coiled-coil peptides. The results reported here also highlight the value of amino acid replacement at multiple sites in peptides and proteins; replacement of a single Leu residue by Hil in T4 lysozyme has been reported to cause an increase of just 1.9°C in the melting temperature of the protein.[2a] In contrast, replacement of six Leu residues in the putative coiled-coil domain of A1 raises T_m by 17°C.

Experimental Section

Detailed experimental protocols can be found in Supporting Information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. a) Liu CC, Schultz PG. Annu Rev Biochem. 2010; 79:413–444. [PubMed: 20307192] b) Dougherty, DA. Protein Engineering. Kohrer, C.; RajBhandary, UL., editors. Springer-Verlag; Berlin: 2009. p. 231-254.c) Budisa, N. Engineering the Genetic Code: Expanding the Amino Acid Repertoire for the Design of Novel Proteins. Wiley-VCH; Weinheim, Germany: 2006. d) Johnson JA, Lu YY, Van Deventer JA, Tirrell DA. Curr Opin Chem Biol. 2010; 14:774–780. [PubMed: 21071259]
2. a) Mendel D, Ellman JA, Chang ZY, Veenstra DL, Kollman PA, Schultz PG. Science. 1992; 256:1798–1802. [PubMed: 1615324] b) Wolschner C, Giese A, Kretzschmar HA, Huber R, Moroder L, Budisa N. Proc Natl Acad Sci USA. 2009; 106:7756–7761. [PubMed: 19416900] c) Montclare JK, Tirrell DA. Angew Chem. 2006; 118:4630–4633.Angew Chem Int Ed. 2006; 45:4518–4521.d) Yoo TH, Link AJ, Tirrell DA. Proc Natl Acad Sci USA. 2007; 104:13887–13890. [PubMed: 17717085] e) Tang Y, Ghirlanda G, Petka WA, Nakajima T, DeGrado WF, Tirrell DA. Angew Chem. 2001; 113:1542–1544.Angew Chem Int Ed. 2001; 40:1494–1496.f) Tang Y, Tirrell DA. J Am Chem Soc. 2001; 123:11089–11090. [PubMed: 11686725] g) Son S, Tanrikulu IC, Tirrell DA. Chem Bio Chem. 2006; 7:1251–1257.h) Montclare JK, Son S, Clark GA, Kumar K, Tirrell DA. Chem Bio Chem. 2009; 10:84–86.
3. Petka WA, Harden JL, McGrath KP, Wirtz D, Tirrell DA. Science. 1998; 281:389–392. [PubMed: 9665877]
4. a) Tang Y, Ghirlanda G, Vaidehi N, Kua J, Mainz DT, Goddard WA, DeGrado WF, Tirrell DA. Biochemistry. 2001; 40:2790–2796. [PubMed: 11258889] b) Bilgicer B, Fichera A, Kumar K. J Am Chem Soc. 2001; 123:4393–4399. [PubMed: 11457223] c) Lee KH, Lee HY, Slutsky MM, Anderson JT, Marsh ENG. Biochemistry. 2004; 43:16277–16284. [PubMed: 15610021]
5. Gao JM, Qiao S, Whitesides GM. J Med Chem. 1995; 38:2292–2301. [PubMed: 7608894]
6. Buer BC, de la Salud-Bea R, Hashimi HMA, Marsh ENG. Biochemistry. 2009; 48:10810–10817. [PubMed: 19824630]
7. Molecular surface areas of Leu, Hil, and (2*S*,4*R*)-Tfl were determined by using Spartan ‘08 (Wavefunction, Inc. Irvine, CA). Structures of amino acids were minimized using Hartree-Fock calculations with a 6–31G* basis in vacuum, followed by determination of the molecular surface areas.
8. a) Boniecki MT, Vu MT, Betha AK, Martinis SA. Proc Natl Acad Sci USA. 2008; 105:19223–19228. [PubMed: 19020078] b) Tang Y, Tirrell DA. Biochemistry. 2002; 41:10635–10645. [PubMed: 12186549]
9. Perez-Iratxeta C, Andrade-Navarro MA. BMC Struct Biol. 2008; 8
10. Tang et al confirmed the predominance of dimers in dilute solutions of A1 containing Leu or fluorinated analogs of Leu at neutral pH; the oligomerization state at low (10 μM) concentrations was found to be insensitive to fluorination.[2e,2f]
11. Schneider JP, Lear JD, DeGrado WF. J Am Chem Soc. 1997; 119:5742–5743.
12. Kwon OH, Yoo TH, Othon CM, Van Deventer JA, Tirrell DA, Zewail AH. Proc Natl Acad Sci USA. 2010; 107:17101–17106. [PubMed: 20855583]

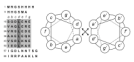

Figure 1.
A1 peptide sequence and helical wheel representation of A1 homodimers. The amino acids that comprise the putative heptad repeats are highlighted in gray, with additional emphasis on the leucine residues at the *d* positions.

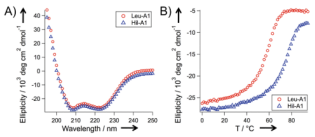

Figure 2.

Circular dichroism spectra of Leu-A1 and Hil-A1. A) Wavelength scans performed at 1°C. B) Ellipticity at 222 nm as a function of temperature. All experiments were performed with 10 mM peptide in PBS, pH 7.4.

Table 1

Kinetic parameters for activation of Leu and Hil by LeuRS.

Substrate[a]	k_{cat} [s^{-1}]	K_m [μM]	k_{cat}/K_m [rel]
Leu[b]	15.1 ± 2.2	3.7 ±1.9	1
Hil	0.4 ± 0.1	77 ± 65	1/690

[a] Leu was used as the L-isomer; Hil as a mixture of the D- and L- isomers. The concentrations of Hil reported here are those of the L-isomer. Kinetic parameters are reported as averages determined from three independent experiments with errors reported as averages of the 95% confidence intervals.

[b] Parameters determined for activation of Leu are consistent with previous reports; the value of k_{cat} measured in this work is within the range of reported values, while the value of K_m reported here is lower than literature values by a factor of 2–10.[2f, 2h, 8]

Table 2

Stabilization of A1 by replacement of Leu with non-canonical amino acids.[a]

Amino acid at *d* position	Leu	(2*S*,4*R*)-Tfl	Hil	Hfl[b]
ΔT_m	0	10[2h]	17	22[2f]

[a] Increase in melting temperature (as compared to Leu-A1) determined from CD spectroscopy of 10 μM solutions of peptide in PBS, pH 7.4.

[b] 74% replacement of Leu.